***N*-(5'-Phosphopyridoxyl)glutamic Acid and *N*-(5'-Phosphopyridoxyl)-2-oxopyrrolidine-5-carboxylic Acid and their Action on the Apoenzyme of Aspartate Aminotransferase**

BY RADII M. KHOMUTOV,* HENRY B. F. DIXON,† LYUDMILA V. VDOVINA,* MIKHAİL P. KIRPICHNIKOV,* YURIİ V. MOROZOV,* EVGENII S. SEVERIN* AND ELENA N. KHURS*

*Institute of Molecular Biology of the Academy of Sciences of the U.S.S.R., Moscow B-312, U.S.S.R., and

†Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. *N*-(5'-Phosphopyridoxyl)-L-glutamic acid (*P*-Pxy-Glu, compound I) is readily converted at pH 3 into a substance (*P*-Pxy-Glp, compound II) characterized as *N*-(5'-phosphopyridoxyl)-2-oxopyrrolidine-5-carboxylic acid. 2. The u.v., i.r. and fluorescence spectra of *P*-Pxy-Glu and *P*-Pxy-Glp have been determined; from the u.v. spectra their *pK* values have been found and compared. 3. The apoenzyme of aspartate aminotransferase is rapidly and irreversibly inactivated by *P*-Pxy-Glu, but is inactivated more slowly by *P*-Pxy-Glp. The complex with *P*-Pxy-Glp is stable enough to be isolated, but it is slowly reactivated in the presence of excess of pyridoxal phosphate. 4. The u.v. spectrum of the complex of apoenzyme and *P*-Pxy-Glp suggests that it contains a hydrogen bond between the phenolic hydroxyl group and the pyrrolidone nitrogen; this specifies the conformation of most of the molecule of *P*-Pxy-Glp. This conformation is similar to that previously postulated for the enzyme-glutamate complex except for the side chain of glutamate. Hence both the affinity of *P*-Pxy-Glp for the apoenzyme and the fact that it is more easily removed than *P*-Pxy-Glu are explicable.

The inhibitory action of *N*-(5'-phosphopyridoxyl)-amino acids on pyridoxal enzymes has been studied because these compounds resemble in their structure the intermediates in the enzymic reaction pathway (Khomutov & Severin, 1969). In particular, *P*-Pxy-Glu† forms a stable inactive complex with the apoenzyme of aspartate aminotransferase (EC 2.6.1.1). Some features of the process of inhibition, such as partial reactivation of the enzyme-inhibitor complex by pyridoxal phosphate, required postulation of inhomogeneity of the inhibitor, possibly because of its easy conversion into another compound. In view of the high effectiveness of this new inhibitor it seemed useful to study in more detail both its properties and its action on the apoenzyme, and the results are presented here.

MATERIALS AND METHODS

N-(5'-Phosphopyridoxyl)-L-glutamic acid (I) was synthesized by a modification of the method of Severin, Gulyaev, Khurs & Khomutov (1969). Pyridoxal 5'-

† Abbreviations: *P*-Pxy-Glu, *N*-(5'-phosphopyridoxyl)-L-glutamic acid (compound I); *P*-Pxy-Glp, *N*-(5'-phosphopyridoxyl)-2-oxopyrrolidine-5-carboxylic acid (compound II).

phosphate hydrate (1 mmol), L-glutamic acid (1.5 mmol) and KOH (7 mmol) were dissolved in methanol (10 ml) and portions of solid KBH_4 were added until the solution was decolorized (about 1 mmol required). The solution was taken to dryness on a rotary evaporator, dissolved in 25 ml of a solution of 0.3 M-acetic acid and 0.3 M-pyridine and chromatographed on a column of DEAE-cellulose (Whatman DE-52) equilibrated and eluted with the same solution. Fig. 1 shows the result. Although the peak of material with extinction at 327 nm is somewhat skew because of overloading, it is adequately separated from other substances. The portions of effluent that contained it were pooled and concentrated to a few ml on a rotary evaporator. Ethanol was added and the evaporation repeated. This was continued until the substance crystallized (yield 0.33 g; 87%). It proved to be identical with the substance previously characterized (Severin *et al.* 1969) as *N*-(5'-phosphopyridoxyl)-L-glutamic acid. It could be recrystallized by dissolving in ethanolic ammonia and evaporating off the NH_3 .

The compound characterized below as *N*-(5'-phosphopyridoxyl)-L-2-oxopyrrolidine-5-carboxylic acid (II) was prepared by dissolving *P*-Pxy-Glu in 0.1 M-acetic acid and heating to 95°C for 5 min. It was crystallized by evaporation and addition of ethanol, like *P*-Pxy-Glu.

Electrophoresis at pH 1.9 was carried out with cooling in white spirit on FN-II or Whatman 3MM paper with a potential gradient of about 100 V/cm in a solution of

Fig. 1. Chromatography of the product from 1 mmol of pyridoxal 5'-phosphate on microgranular DEAE-cellulose ($36 \text{ cm} \times 7.1 \text{ cm}^2$) equilibrated and eluted with 0.3 M-acetic acid and 0.3 M-pyridine at 1 ml/min per cm^2 .

formic acid (2%, v/v) and acetic acid (8%, v/v). For pH 3.5 the solution was pyridine (1%, v/v) and acetic acid (10%, v/v). After evaporation of the solvent the paper was lightly sprayed with a 1% solution of 2,6-dichloro-*p*-benzoquinone-4-chloroimine in ethanol, dried in air and exposed to an atmosphere of NH_3 until bright blue spots appeared. Glucose was used as a marker to correct for electro-osmosis.

The apoenzyme of aspartate aminotransferase was prepared by the method of Dixon & Severin (1968) and its potential activity was measured by the method of Severin & Dixon (1968). The enzyme-inhibitor complexes were prepared by treating a solution of the apoenzyme (4 mg/ml, i.e. $100 \mu\text{M}$, calculated on subunits) with inhibitor (1 mol/mol in the case of compound *P*-Pxy-Glu, and 3 mol/mol in the case of *P*-Pxy-Glp). After 15 min the degree of inhibition was measured, and found to be complete for *P*-Pxy-Glu, but this required about 3 h for *P*-Pxy-Glp. When the inhibition was complete, the enzyme-inhibitor complex was passed through a column of Sephadex G-25 equilibrated with water. The solution was adjusted to pH 5 with NaOH or HCl and the spectra of the complexes measured (Fig. 2).

Absorption spectra were measured with a double-beam recording spectrophotometer (Hitachi, Japan, model EPS-2). Circular dichroism was measured with a Jouan Dichrograph. Fluorescent spectra and quantum yields of emission were measured with the apparatus described by Morozov & Tumerman (1967). The spectra are corrected, i.e. the spectral sensitivity of the apparatus was determined. The absolute quantum yields of the compounds studied were determined in a light-scattering sphere relative to a standard of quinine sulphate, whose absolute quantum yield is 0.55 at 25°C , with the subsequent introduction of spectral corrections by a method similar to that of Chen (1965). All fluorescence measurements were carried out at a concentration of $100 \mu\text{M}$. For substances of the vitamin B_6 group there is no concentration-dependent quenching of fluorescence, at

least up to a concentration of 1 mM, because of the small overlap integral between extinction and fluorescence spectra.

Individual absorption spectra of the various ionic forms of the compounds and the pK values for the inter-conversion of these forms were calculated from the spectra obtained at various pH values by using a computer program whose algorithm is described by Bazhulina, Lomakin, Morozov & Savin (1971).

Before measurement of the i.r. spectra, *P*-Pxy-Glu and *P*-Pxy-Glp were repurified by preparative electrophoresis on paper at pH 1.9 with subsequent freeze-drying. The spectra of the cationic forms were then measured in the solid state (with KBr).

RESULTS AND DISCUSSION

The synthesis of compound (I) (*P*-Pxy-Glu) that was described earlier yielded a homogeneous substance, as was shown by electrophoresis at pH 3.5 (Severin *et al.* 1969). Further purification by chromatography on DEAE-cellulose did not change its electrophoretic mobility. We observed, however, that freshly prepared and old samples of the compound differed markedly in their inhibitory action on the apotransaminase. Electrophoresis showed that the old samples contained two substances that differed in mobility. In addition to the spot shown in fresh samples, there was another (compound II). Brief heating of compound (I) in acid solution, or a long incubation at room temperature at pH 2 (a control for peptic hydrolysis of the enzyme-inhibitor complex) converted compound (I) into compound (II) with changed mobility and with only weak fluorescence. The two compounds were also separated by chromatography on DEAE-cellulose (Fig. 3).

Both compounds gave the reaction with 2,6-dichloro-*p*-benzoquinone-4-chloroimine that is characteristic for phenols with unsubstituted *para*-positions, and they had similar spectra at pH values of 1, 7 and 13. They differed significantly, however, in their action on the apotransaminase. Compound (I) rapidly and irreversibly inhibited the apoenzyme, so that activity was not restored with coenzyme, and the complex formed had a marked dichroism at 330 nm. In contrast, only a long (3 h) incubation of the apoenzyme with an excess of compound (II) gave inhibition, and the enzyme-inhibitor complex was reactivated during a 2-3 h incubation with the 1000-fold excess of coenzyme used for measurement of the potential activity of the apoenzyme. This complex exhibited only weak dichroism at 330 nm. We are hoping to publish separately the results of a more detailed study of these enzyme-inhibitor complexes.

To elucidate the structures of compounds (I) and (II) we examined their absorption spectra and fluorescent characteristics at various pH values.

Fig. 2. Spectra of the complexes ($100\mu\text{M}$) between the apoenzyme and (a) *P*-Pxy-Glu and (b) *P*-Pxy-Glp. The apoenzyme was treated with inhibitor until it was inactivated (see the Materials and Methods section) and then gel filtered into water before the spectra were recorded. The broken lines show the spectra of the complex with *P*-Pxy-Glp after treatment with $100\mu\text{M}$ -pyridoxal 5'-phosphate for 24h and gel filtration. The spectra of the complex with *P*-Pxy-Glu were unchanged by similar treatment with pyridoxal 5'-phosphate.

Fig. 3. Chromatography of a mixture of pyridoxine 5'-phosphate (PNP), *P*-Pxy-Glu (I) and *P*-Pxy-Glp (II) (5mg of each) on microgranular DEAE-cellulose ($53\text{cm} \times 1.03\text{cm}^2$) equilibrated and eluted with 0.3M -acetic acid and 0.3M -pyridine at 1.3ml/min per cm^2 . —, E_{327} ; ---, E_{295} .

The absorption spectra of compound (I) change with pH in almost the same way as those of pyridoxamine 5'-phosphate (Figs. 4a and 4b; cf. Bazhulina *et al.* 1971). In the region from pH 1 to pH 6 sharp isosbestic points are found, and this is evidence of the equilibrium of only two ionic forms; in the interval pH 7–13 there are two subregions (pH 8–10 and pH 10–13), which suggests the equilibrium of three ionic forms. This fact together with the identity of the spectral shifts and of their order of appearance with changing pH allows us to conclude that in both cases the spectral changes arise from the ionization of analogous groups: the phenolic hydroxyl group, the pyridine nitrogen, and the nitrogen on the 4'-carbon. The ionizations of the phosphate and carboxyl groups are not reflected in the absorption spectra of *P*-Pxy-Glu.

The absorption spectra of compound (II) (Figs. 4c and 4d) show two main differences from those of compound (I). For compound (II) the whole pH range 1–13 may be divided into two regions pH 1–8 and pH 9–13, in each of which sharp isosbestic points are observed. Thus in contrast with *P*-Pxy-Glu, which has four spectrally distinct ionic forms, compound (II) has only three. Further, comparison of Fig. 4(a) with Fig. 4(c) shows that the pK of the

These methods were chosen partly because they could also be used in the further study of the corresponding enzyme-inhibitor complexes.

Fig. 4. Observed spectra of *P*-Pxy-Glu (a and b) and of *P*-Pxy-Glp (c and d).

phenolic hydroxyl group of compound (I) has a value just over 3, whereas that of compound (II) is just below 6.

Comparison of the computed spectra of the individual ionic forms (Fig. 5) shows that in each case loss of the first proton leads to a shift of the maximum to longer wavelengths by 30–35 nm. This large shift must be ascribed to ionization of the phenolic hydroxyl group (Scheme 1, Ia→Ib and IIa→IIb). Loss of the second proton evokes, in both cases, a shift to shorter wavelengths by 16–18 nm, which must be ascribed to deprotonation of the pyridine nitrogen (Ib→Ic and IIb→IIc). Finally, deprotonation of the 4'-nitrogen, which takes place only for *P*-Pxy-Glu (Ic→Id), is accompanied by a shift to shorter wavelengths of 2–3 nm. At neutral and alkaline pH values compound (II) is characterized by a shift to longer wavelengths of 2–4 nm in comparison with compound (I), whereas in the acid region it has a shift to shorter wavelengths of 2 nm. The content of the neutral forms [the tautomers of compounds (Ib) and (IIb) with extinction maxima at 290 nm] is significantly higher for compound (II) than for compound (I), as expected from the fact that, for the former, the first and second p*K* values are closer together. Similarly the tautomer of compound (Ic) with the proton moved from the 4'-nitrogen to the ring-nitrogen is likely to contribute significantly in view of the closeness of the last two p*K* values; this may explain the width of its absorption band (Fig. 5).

Scheme 1 lists the p*K* values calculated. From these results compound (II) is most clearly characterized by the absence of dissociation of a proton from the 4'-nitrogen, and also by the marked rise in p*K* for both the phenolic hydroxyl group and the pyridine nitrogen.

In view of the known ease of cyclization of glutamic acid in acid solutions to form pyrrolidone-carboxylic acid, it seemed likely that compound (II) was *N*-(5'-phosphopyridoxyl)-2-oxopyrrolidine-5-carboxylic acid (*P*-Pxy-Glp). Ikawa (1967) reported the formation of this compound on reduction of the phosphopyridoxylidene derivative of glutamic acid, but did not present evidence of its structure. As a result of formation of the lactam structure, protonation of the 4'-nitrogen will occur only below pH 1. The resulting absence of a charge on this nitrogen may by itself lead to the changes in p*K*₁ and p*K*₂ of *P*-Pxy-Glp in comparison with *P*-Pxy-Glu. Further, there could be some supplementary stabilization of the proton on the phenolic hydroxyl group by intramolecular hydrogen-bonding (Scheme 1).

A hydrogen bond involving the proton of the phenolic hydroxyl group could be formed either with the carbonyl oxygen or with the amide nitrogen of the lactam. Examination of models shows that in the first case a strained eight-membered ring is formed, in which the α-N-4'-C bond must be orientated in a plane at right angles to the plane of the pyridine ring (Fig. 6a). We prefer

Fig. 5. Computed spectra of the various ionic forms (Scheme 1) of (a) *P*-Pxy-Glu and (b) *P*-Pxy-Glp.

the other structure in which the hydrogen bond to the lactam nitrogen forms a six-membered ring (Fig. 6b), which is more likely to be stable. This system is analogous to the structure of the Schiff base formed between pyridoxal phosphate and amino acids, which is stabilized by intramolecular hydrogen bonding between the phenolic hydroxyl group and the imine nitrogen.

Both the formation of the lactam structure and the presence of the intramolecular hydrogen bond can be directly checked, at least in the solid state, by i.r. spectroscopy. The i.r. spectra of both compounds showed the frequencies characteristic of carboxylate ions. In the i.r. spectrum of *P*-Pxy-Glp, in contrast with that of *P*-Pxy-Glu, an intense band was observed at 1675 cm^{-1} , which can be ascribed to the stretching of the carbonyl group (the amide I band, cf. Weber & Teale, 1965), and a comparatively wide band in the region of 3200 cm^{-1} , corresponding to the vibration of a hydroxyl group that participates in an intramolecular hydrogen bond.

At pH 1.9 the electrophoretic mobilities of *P*-Pxy-Glu and *P*-Pxy-Glp were $+1.4 \times 10^{-3}$ and $-0.8 \times 10^{-3}\text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{min}^{-1}$ respectively, and at pH 3.5 they were -0.4×10^{-3} and $-5.0 \times 10^{-3}\text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{min}^{-1}$ respectively. The fact that *P*-Pxy-Glu is isoelectric only just above pH 2 explains why the substance when isolated in its isoelectric

form is acidic enough to be easily converted on storage into *P*-Pxy-Glp. If the phosphate groups of both compounds have approximately equal $\text{p}K_1$ values, and the carboxyl groups of *P*-Pxy-Glu approximately the same dissociation constants of those of glutamic acid, it is necessary to suppose that the carboxyl group of *P*-Pxy-Glp has a $\text{p}K$ of well below 3. This is first because the group must be appreciably ionized at pH 1.9 to explain how *P*-Pxy-Glp can be negatively charged at this pH when *P*-Pxy-Glu is positive. Secondly the carboxyl group must be almost completely charged at pH 3.5 to explain why *P*-Pxy-Glp has considerably greater mobility than *P*-Pxy-Glu at this pH, even though its lower molecular weight and more compact structure (Fig. 7) assist in this respect. The fact that this carboxyl group is unusually acidic for an *N*-acylated α -amino acid (cf. pyrrolidone carboxylic acid whose $\text{p}K$ is 3.3) may be a consequence of the involvement of the amide nitrogen in hydrogen bonding.

The combination of several experimental results and the consideration of models thus leads to a spatial structure for *P*-Pxy-Glp such that the supplementary six-membered ring produced by the intramolecular hydrogen bond forms a single coplanar system with the pyridine ring. In this structure (Fig. 6b) the lactam ring is orientated perpendicularly with respect to the plane of the pyridine ring, and the α -carboxyl group can be directed to the side of the substituent on the 5'-position. It seems also that the phosphate group can approach the α -nitrogen, the δ -carbon or both, as evidenced by the ease of cyclization of *P*-Pxy-Glu into *P*-Pxy-Glp (completion of the reaction in 5 min at pH 3 and 95°C) in comparison with the analogous conversion of Pxy-Glu (for which no detectable quantities of product are formed under these conditions).

The fluorescence spectra and quantum yields have also been measured for the various ionic forms of *P*-Pxy-Glu and *P*-Pxy-Glp. As for many other derivatives of vitamin B₆ (Morozov, Bazhulina & Karpeisky, 1968), excitation of the cationic forms of both compounds leads to a photo-ionization of the phenolic hydroxyl group, so that the ions (Ib) and (IIb) (Scheme 1) prove responsible for the fluorescence (Fig. 8). Thus the intramolecular hydrogen bond may not exist in the excited state. The small, but significant, displacement of the emission spectra in the neutral region compared with those in acid is connected with the ionization of the phosphate group (Morozov *et al.* 1968). Table 1 shows the quantum yields of fluorescence of *P*-Pxy-Glu and *P*-Pxy-Glp at various pH values. The marked difference in quantum yield, particularly at pH 1–7, provides an analytical method for distinguishing *P*-Pxy-Glu and *P*-Pxy-Glp.

Scheme 1. Possible ionic forms of compounds (I) and (II), with the wavelengths of their extinction maxima. R = $\text{CH}_2\text{-O-PO}_3\text{H}_2$ and deprotonated forms; R' = $\text{CH}(\text{CO}_2\text{H})\text{-(CH}_2)_2\text{-CO}_2\text{H}$ and deprotonated forms; R'' = CO_2H and CO_2^- . The pK values in brackets are those found for pyridoxamine 5'-phosphate (R' = H) by Bazhulina *et al.* (1971).

Fig. 6. Possible conformations of P-Pxy-Glp with hydrogen bonding of the phenolic hydroxyl group to (a) the carbonyl oxygen and (b) the amide nitrogen.

Fig. 7. Postulated ionic forms of *P*-Pxy-Glu and *P*-Pxy-Glp at pH 3.5. Fig. 8. Fluorescence spectra of (a) *P*-Pxy-Glu and (b) *P*-Pxy-Glp, with excitation at 313 nm.

Table 1. Quantum yields of fluorescence

pH	<i>P</i> -Pxy-Glu	<i>P</i> -Pxy-Glp
1.0	0.17 ± 0.03	0.05 ± 0.01
6.0	0.19 ± 0.03	
8.0		0.06 ± 0.015
10.0	0.04 ± 0.01*	
12.0	0.10 ± 0.02	0.14 ± 0.03

* Total quantum yield of all the ionic forms.

Fig. 9. Dependence of the apparent rate constant for inactivation of the apoenzyme by *P*-Pxy-Glu upon the concentration of this inhibitor.

The complex of apoenzyme with *P*-Pxy-Glu was treated with an equivalent amount of pyridoxal phosphate at pH 5.4, but this did not give any reconstruction of the native enzyme (Fig. 2a). When, however, the complex of the apoenzyme with *P*-Pxy-Glp was similarly treated, the pyridoxal phosphate slowly displaced the *P*-Pxy-Glp from the protein and reconstituted the native enzyme, as

judged by the rise in both extinction and circular dichroism at 430 nm (Fig. 2b). Analogous results were obtained by treating the complexes (100 nm) with 100 μM-pyridoxal phosphate in tris-HCl buffer at pH 8.3 and judging reconstitution by the return

of activity; no reactivation was observed for the complex with *P*-Pxy-Glu, but a slow one leading to 35% reactivation in 24h was observed for the complex with *P*-Pxy-Glp.

The rate of combination of apoenzyme with *P*-Pxy-Glu was studied by incubating it at a concentration of $1.25\ \mu\text{M}$ in 0.25M -tris-HCl buffer, pH 7.4, at 25°C with the inhibitor at concentrations of 12.5 – $50\ \mu\text{M}$. Residual potential activity was measured by the method of Severin & Dixon (1968) at 5min intervals for 30 min. From the pseudo-first-order rate constants observed, the rate of inactivation was found to obey saturation kinetics (Fig. 9; cf. Kitz & Wilson, 1962; Shaw & Glover, 1970). This allowed calculation of the K_i for the reversible part of the reaction as $28\ \mu\text{M}$ and the rate constant for the irreversible part as $0.13\ \text{min}^{-1}$.

Comparison of the absorption spectra of the complexes with apoenzyme of *P*-Pxy-Glu and *P*-Pxy-Glp with those of the free compounds allows some conclusions to be drawn about steric relationships in the active centre of aspartate aminotransferase. When *P*-Pxy-Glp interacts with the apoenzyme at pH 5 (Fig. 2), no increase in E_{330} is observed. This suggests that the enzyme evokes no breakage of the intramolecular hydrogen bond (Fig. 6b), which agrees with the known existence of stable hydrogen bonds in the enzyme. We consequently consider that the conformation of the molecules of free *P*-Pxy-Glp as they exist in solution is largely conserved in the enzyme-inhibitor complex. On this supposition we note that the α -carboxyl group can occupy approximately the same position with respect to the coenzyme (directed towards the substituent in the 5'-position) that follows from the results on the inhibition of aspartate aminotransferase by the cycloglutamates (Khomutov, Kovaleva, Severin & Vdovina, 1967). We do not know if the phosphate and carboxyl groups are close in enzyme-bound *P*-Pxy-Glp; the rate of dephosphorylation of the complex is insignificant like that of the free compound.

Thus we may suppose that the configuration of *P*-Pxy-Glp is fairly close to that of the active centre of aspartate aminotransferase, although lack of the γ -carboxyl group and the fixing of the side chain of glutamic acid in the form of the lactam significantly lower the affinity of the inhibitor for the apoenzyme. This agrees with the facts that *P*-Pxy-Glu, when bound to the apoenzyme, does not undergo conversion into *P*-Pxy-Glp even on prolonged incubation, and that the actual conformation of glutamic acid in the active centre of the enzyme is characterized by separation of the γ -carboxyl group from the α -amino group (Khomutov & Severin, 1969).

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